Potent *ex vivo* Expanded, Human Placental CD34$^+$-derived Natural Killer Cells for Glioblastoma Immunotherapy

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INTRODUCTION

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults (Gittleman, 2018). With intensive research efforts and a multimodal management that consists of surgery, radiotherapy and chemotherapy with temozolomide, the prognosis remains very poor with 5-year survival rate of less than 10% (Kamiya-Matsuoka, 2015). Therefore, there is an unmet medical need to develop novel treatments for GBM.

It has been demonstrated that *ex vivo* expanded human peripheral blood NK (PBNK) cells from healthy donors exhibited *in vitro* and *in vivo* anti-tumor activities against patient derived GBM cells (Lee, 2015). It was also reported that both CD133- medulloblastoma cells and CD133+ cancer stem cells were susceptible to NK cell-mediated killing *in vitro* (Castriconi, 2007). Ishikawa et al (2004) reported Phase I data for an intracranial and intravenous autologous NK cell injection that showed tumor regression in a small number of patients. Several ongoing clinical trials (ClinicalTrial.gov: NCT03360708, NCT01588769, NCT00331526, NCT03383978, NCT00003067) are investigating the potential of adoptive NK cell immunotherapy for GBM.

Celularity has established an *ex vivo* process to generate Placental derived intermediate Nature Killer (PiNK) cells from placental CD34$^+$ cells with an average of 1.0 x 10^5 fold expansion and ~90% purity of CD56$^+$CD3$^-$ cells. Here, we have demonstrated that PiNK cells exhibited *in vitro* cytotoxicity against GBM cell lines and cytokine secretion activity following exposure to tumor cells. *In vivo* efficacy studies further demonstrated the activity of PiNK against tumor growth in a U-87MG orthotopic NSG mouse model. Taken together, our data support the application of PiNK cells for the development of an allogeneic adoptive immunotherapeutic for patients with GBM.

METHODS

***Ex vivo* expansion of PiNK cells**: Placental CD34$^+$ cells were cultivated in the presence of cytokines including thromobopoietin, SCF, Flt3 ligand, IL-7, IL-15 and IL-2 for 35 days to generate PiNK cells.

Phenotype characterizations of PiNK cells: PiNK cells were subjected to phenotypic characterization via multi-color flow cytometry using a FACSCanto II (BD Biosciences). The data analysis was performed using FlowJo software (TreeStar).

***In vitro* cytotoxicity of PiNK cells against GBM cells:** The cytotoxicity of PiNK cells against tumor cell lines was assessed by a PKH26/TO-PRO-3 FACS based assay (4-hour assay). A range of effector to target (E:T) ratios were assessed as indicated. Tumor cell lines including U-251, LN-18, U-87 MG, U-118 MG and K562 were obtained from ATCC. K562 was used as assay control. To dissect the mechanism of PiNK cells against GBM cells, the PiNK cells were pretreated with 10μg/mL blocking antibodies as indicated or corresponding isotype control; or 50nM perforin inhibitor (Concanamycin A, CMA), or corresponding control at 5% CO_2, 37^{0}C for 30min, followed by 4h cytotoxicity assay against U-87 MG cells. The percentage of cytotoxicity change was reported as: (Cytotoxicity by blocking antibodies/perforin inhibitor (CMA) – Cytotoxicty by control) / Cytotoxicty by control * 100%.

***In vitro* cytokine secretion of PiNK in the presence of GBM cells:** PiNK cells (1x10^5 cells) were incubated with GBM cells (1x10^5 cells) in 96-well U-bottom tissue culture plates at an E:T ratio of 1:1 for 24 hours. After incubation, the supernatant was collected and cytokine concentrations were determined by Luminex analysis using MILLIPLEX MAP magnetic bead kits according to the protocol provided by the manufacturer (EMD Millipore). The data were analyzed using Milliplex Xponent and Analyst software.

***In vivo* U-87 MG orthotopic NSG mouse model:** Luciferase-expressing U-87 MG cells (1x10^4) were stereotactically injected into the cranium of NSG mice at Day0. Single dosing of 0.5x10^6 PiNK at Day14 or repeated dosing of 0.5x10^6 PiNK at Day14 and Day21 by intracranial injection (IC) were administered. Live bioluminescence imaging (BLI) were taken twice per week until study termination. In addition, clinical symptoms were monitored and body weight were measured weekly. The data were analyzed by GraphPad Prism.

RESULTS

Ex Vivo Cultivation Procedure Established to Produce PiNK Cells

Celularity has established an *ex vivo* process to generate PiNK cells from placental CD34$^+$ cells with an average of 1.0 x 10^5 fold expansion and 88.3% ± 6.3% purity of CD56$^+$CD3$^-$ cells (n=20 donors) (Figure 1A, 1B). PiNK cells expressed similar percentages of NKG2D, NKp46, NKp30, CD94, CD226 and 2B4, higher percentage of NKp44, and lower percentage of CD16 and KIRs in comparison to NK cells derived from PB (Figure 1C).

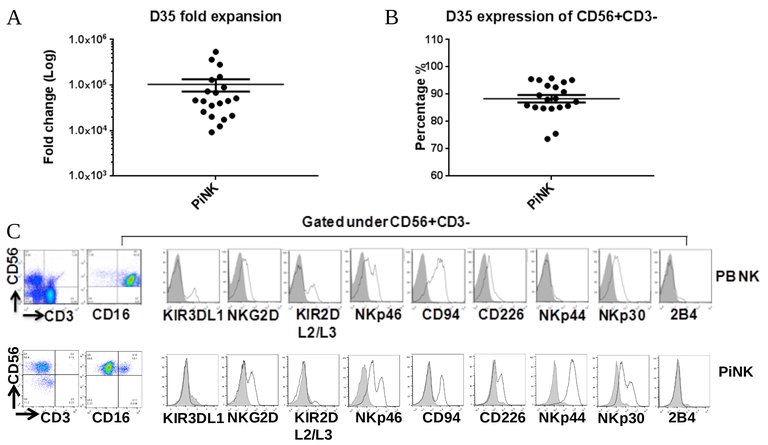

Figure 1. Fold expansion, purity and phenotype characterization of placental CD34$^+$ derived PiNK cells. A) Fold expansion. B) Purity by percentage of CD56$^+$CD3$^-$ of *ex vivo* expanded PiNK cells (n=20 donors). C) Expression of NK surface markers of PiNK cells in comparison with PB NK cells under the gate of CD56$^+$CD3$^-$.

In Vitro Anti-tumor Activity of PiNK Against GBM Cells

PiNK cells (n=6 donors) exhibited 59.4%±1.5%, 47.6%±10.5%, 37.7%±12.3%, and 8.5%±3.9% cytotoxicity against U-251, LN-18, U-87 MG and U-118 MG GBM cell lines, respectively, in a 4-h cytotoxicity assay at E:T ratio of 10:1 (Figure 2A). PiNK cells did not show any cytotoxic activity against PBMCs from unrelated healthy donors at any of the E:T ratios tested up to 100:1 (Figure 2B), indicating that PiNK cells were capable not only of lysing tumor cells, but also of discriminating between healthy and tumor targets. PiNK cells secreted immunomodulatory cytokines, namely IFN-γ, TNF-α, and Granzyme B, in the presence of GBM cell lines tested (Figure 3). By using blocking antibodies and/or perforin inhibitor (CMA), we have identified that Perforin or TRAIL, or a combination of them; NKG2D or DNAM-1, or a combination of them; played an important role in PiNK-mediated cytotoxicity against U-87 MG (Figure 4A, 4B).

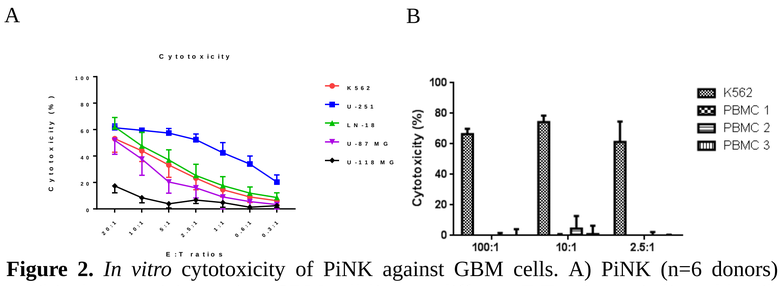

Figure 2. *In vitro* cytotoxicity of PiNK against GBM cells. A) PiNK (n=6 donors) exhibited cytotoxicity against GBM cell lines at different E:T ratios as indicated by 4h cytotoxicity assay. B) PiNK (n=3 donors) displayed no cytotoxicity against unrelated healthy PBMC donors (n=3 donors).

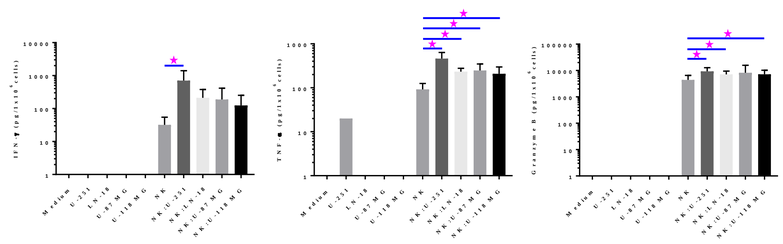

Figure 3. PiNK cells secreted IFN-γ, TNF-α and Granzyme B in the presence of GBM cells at an E:T ratio of 1:1 for 24h. The error bars represent the standard deviation calculated from the mean of 5 PiNK donors (P<0.05).

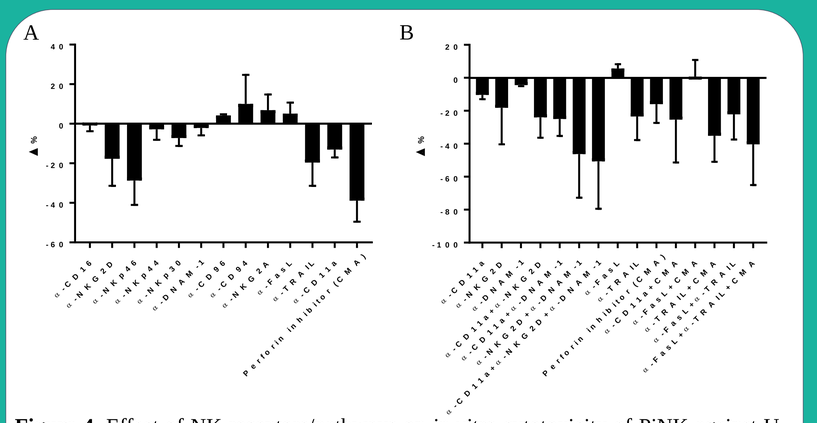

Figure 4. Effect of NK receptors/pathways on *in vitro* cytotoxicity of PiNK against U-87 MG cells by antibody blocking or perforin inhibitor. A) Change of cytotoxicity of PiNK cells against U-87 MG cells at an E:T ratio of 10:1 from single antibody blocking/perforin inhibitor as indicated vs. PiNK cells by corresponding control (n=3 donors). B) Change of cytotoxicity of PiNK cells against U-87 MG cells at an E:T ratio of 10:1 from combination antibody blocking/perforin inhibitor as indicated vs. PiNK cells by corresponding control (n=3 donors).

In Vivo Anti-tumor Activity of PiNK Cells Against Orthotopic U-87 MG NSG Mouse Model

The U-87 MG orthotopic mouse model was used to assess *in vivo* anti-GBM activity of PiNK cells. IC with single dosing of 0.5x10^6 PiNK at Day14 or repeated dosing of 0.5x10^6 PiNK at Day14 and Day21 were evaluated. No abnormal clinical symptoms were observed in the animals with either single or two repeated IC injections of 0.5x10^6 PiNK cells. PiNK cells with single (n=6 mice per group) or repeated dosing (n=3 mice per group), significantly reduced BLI signal on Day 25, 28 and 35 compared with the PBS control (n=6 mice per group) (P<0.05). Furthermore, PiNK cells with repeated IC dosing significantly reduced BLI signal on Day25, 28 and 35 compared with PiNK single IC dosing (P<0.05) (Figure 5A, 5B).

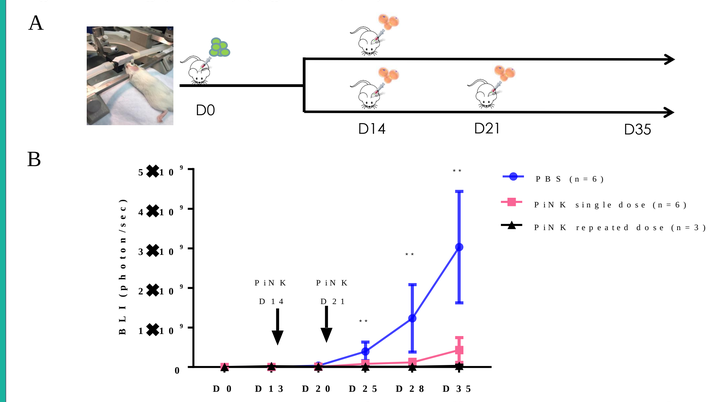

Figure 5. *In vivo* anti-tumor activity of PiNK cells against U-87 MG orthotopic NSG mouse model. A) 1×10^4 luciferase-expressing U-87 MG cells were stereotactically injected into the cranium of NSG mice at Day0. Single dosing of 0.5x10^6 PiNK at Day14 or repeated dosing of 0.5x10^6 PiNK at Day14 and Day21 were administered by intracranial injection (IC). B) PiNK cells with single dosing (n=6 mice per group) or repeated dosing (n=3 mice per group) significantly reduced tumor growth compared with vehicle control (n=6 mice per group). PiNK cells with repeated dosing significantly reduced BLI compared with single dosing (P<0.05).

CONCLUSION

Celularity has established an *ex vivo* process to generate PiNK cells from placental CD34$^+$ cells with an average of 1.0 x 10^5 fold expansion and ~90% purity of CD56$^+$CD3$^-$ cells. PiNK cells exhibited *in vitro* cytotoxic and cytokine secreting activities against several GBM tumor cell lines, whereas PiNK cells showed no cytotoxicity against unrelated healthy PBMC. In addition, our data demonstrated that Perforin, TRAIL, NKG2D and DNAM-1 played important roles in PiNK-mediated cytotoxicity against U-87 MG. Furthermore, *in vivo* anti-tumor activity of PiNK was demonstrated in U-87 MG orthotopic NSG mouse model. Taken together, our data support the development of PiNK for the treatment of GBM.

REFERENCE

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